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Human Herpesvirus 6 in Human Immunodeficiency Virus-Infected Individuals: Association With Early Histologic Phases of Lymphadenopathy Syndrome But Not With Malignant Lymphoproliferative Disorders

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Preliminary evidence suggested that human herpesvirus-6 (HHV-6) may act as a cofactor in acquired immunodeficiency syndrome (AIDS) and may contribute to the pathogenesis of lymphoproliferative disorders occurring in individuals infected with the human immunodeficiency virus (HIV). To understand better the biological and clinical significance of HHV-6 infection in the context of HIV-related immunosuppression, the polymerase chain reaction was used to study the frequency and variant distribution of HHV-6 in peripheral blood mononucleated cells (PBMCs) from HIV-seropositive individuals, either asymptomatic or with lymphadenopathy syndrome (LAS) or with overt AIDS. Non-neoplastic and malignant lymphoproliferative disorders from both HIV-infected and HIV-seronegative patients were also investigated using the same series of samples for the presence of Epstein-Barr virus (EBV). When compared with healthy blood donors (12/42, 29%), HHV-6 prevalence in PBMCs showed a progressive decline in HIV-seropositive individuals with asymptomatic HIV infection (3/26, 11%) and in patients with LAS (1/13, 8%) and a significant reduction in patients with overt AIDS (1/20, 20%; $P = 0.02$). The decrease correlated with the number of $CD4^+$ cells at the time of examination. In addition, HHV-6 DNA sequences were significantly more prevalent in LAS biopsies (13/20, 65%) than in HIV-unrelated reactive lymphadenopathies (2/10, 20%; $P = 0.02$) and the presence of HHV-6 sequences correlated closely with a histologic pattern of follicular hyperplasia (13/16, 81%; $P = 0.003$). Strikingly, HHV-6 prevalence decreased in PBMCs of LAS patients, suggesting that the likelihood of interactions between HHV-6 and HIV varies in different body districts. In particular, the demonstration that all HHV-6-carrying LAS samples were also positive for HIV infection suggests that LAS lymph nodes constitute one of the sites where biologically relevant interactions between the two viruses might occur. Also, the prevalence of EBV was higher in LAS (14/20, 70%) than in non-neoplastic lymph nodes from HIV-seronegative individuals (4/10, 40%), although the difference was not statistically significant. EBV was associated strongly with HIV-related malignant lymphoproliferative disorders, being detected in 100% of patients with Hodgkin's disease (HD) and 53% of B-cell non-Hodgkin's lymphomas (NHL). In contrast, the prevalence of HHV-6 DNA in HD and B-cell NHL arisen in HIV-infected patients (30% and 6%, respectively) was remarkably lower and similar to that observed in lymphoproliferative disorders from HIV-seronegative patients. Finally, as observed in healthy individuals, HHV-6 variant B was more prevalent than variant A in benign and malignant lymphoproliferative disorders from both HIV-infected and HIV-seronegative patients. These results suggest that the interactions between HHV-6 and HIV could be different in the various phases of HIV disease and in different districts; HHV-6 has probably no direct role in the pathogenesis of HIV-associated B-cell NHL and HD cases, and behave differently from EBV; and HIV-related immunosuppression does not

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alter the distribution of HHV-6 variants in these tissues, as observed in the case of EBV.

KEY WORDS: human herpesvirus-6, Epstein-Barr virus, HIV infection, AIDS, malignant lymphomas, PCR

INTRODUCTION

Human herpesvirus-6 (HHV-6) is a primarily T-lymphotropic herpesvirus, isolated originally from peripheral blood mononuclear cells (PBMCs) of patients with lymphoproliferative disorders or acquired immunodeficiency syndrome (AIDS) [Salahuddin et al., 1986]. HHV-6 infection is widespread in the human population, with a seroprevalence exceeding 90% [Saxinger et al., 1988; Okuno et al., 1989]. Primary infection occurs early in life and is inapparent or causes exanthem subitum (roseola infantum), a common febrile illness with rash in infancy [Yamanishi et al., 1988]. HHV-6 is also linked with self-limiting diseases such as mononucleosis-like illness [Akashi et al., 1993] and acute hepatitis [Dubedat and Kappagoda, 1989], and anecdotal reports suggest that lytic HHV-6 infection in adults may be associated with chronic fatigue syndrome, afebrile lymphadenopathy, meningoencephalitis and a hemophagocytic syndrome [reviewed in Pellett et al., 1992]. On the basis of different molecular, biological and immunological properties, two distinct HHV-6 variants, A and B, have been identified recently [Ablashi et al., 1991, 1993]. All HHV-6 strains isolated from children with exanthem subitum are so far HHV-6 B; only in two cases were both variants isolated [Ablashi et al., 1993]. HHV-6 from immunocompromised patients may belong to either variant [Ablashi et al., 1991, 1993]. It was reported recently that HHV-6 A but not HHV-6 B is detected frequently in Kaposi's sarcoma biopsies, suggesting that the two variants may be associated with different clinico-pathologic entities or show a different tropism [Bovenzi et al., 1993].

Although recent reports have provided suggestive evidence for an in vitro-transforming potential of HHV-6 [Razzaque, 1990], the role of this virus in the pathogenesis of lymphoproliferative disorders is still obscure. HHV-6 DNA sequences have been detected so far at low frequencies in Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) biopsies [reviewed by Pellett et al., 1992], suggesting that HHV-6 may have a direct etiological role only in a fraction of cases [Di Luca et al., 1994]. On the other hand, it has also been suggested that, as a consequence of persistent active HHV-6 infections, the virus might contribute to the development of lymphoproliferative disorders by stimulating polyclonal B-cell activation, thus enlarging the pool of cells susceptible for further transforming events [Krueger et al., 1989].

In analogy with other herpesviruses, HHV-6 is responsible for latent, lifelong infection of the host and may be reactivated during immunosuppression [Okuno et al., 1990; Carrigan et al., 1991]. Increased HHV-6 antibody titers, probably following virus reactivation, have been reported in patients with HD, African Burkitt's lymphoma, Sjögren's syndrome and other diseases with underlying impairment of cellular immune responses [Ablashi et al., 1988; Biberfeld et al., 1988; Clark et al., 1990]. HHV-6 reactivation has also been suspected to occur in the context of human immunodeficiency virus-1 (HIV-1)-related immunosuppression [Knox and Carrigan, 1994]. Nevertheless, due to limited reliability of serological tests currently available for HHV-6 and the extensive spread of the virus in the healthy population [Saxinger et al., 1988; Okuno et al., 1989], the seroepidemiological studies performed so far failed to clearly assess whether HHV-6 reactivation occurs in the HIV setting and whether HHV-6 is able to affect the course of HIV infection [Spira et al., 1990; Chen et al., 1992]. The hypothesis that HHV-6 may be a cofactor in the evolution of HIV infection is based on several lines of evidence, including the frequent isolation of HHV-6 from AIDS patients [Salahuddin et al., 1986] and the demonstration that this virus has a preferential tropism for $CD4^+$ T-lymphocytes [Lusso et al., 1988], is able to transactivate HIV regulatory elements [Horvat et al., 1989; Lusso et al., 1989], can increase HIV replication [Ensoli et al., 1989] and positively synergizes with HIV in the induction of cytopathic effects in coinfected cells [Lusso et al., 1989]. Recently, the use of more specific and sensitive analytical methods allowed the detection of HHV-6 genomes and virus-encoded antigens in a variety of tissues taken at necropsy from AIDS patients, suggesting that, at least in the late phases of the disease, HHV-6 infection is active and disseminated [Corbellino et al., 1993; Knox and Carrigan, 1994]. Nevertheless, it is not yet clear whether HHV-6 might influence the early phases of HIV disease and in particular, whether it might contribute to the progression to overt AIDS. In addition, no information is available on the possible pathogenetic association between HHV-6 and the lymphoproliferative disorders arising in HIV-infected individuals.

In order to understand the clinical significance of HHV-6 infection in the setting of HIV-1-related immunosuppression, the polymerase chain reaction (PCR) was used to investigate the frequency and variant distribution of HHV-6 in PBMCs from individuals with asymptomatic HIV infection, lymphadenopathy syndrome (LAS) and overt AIDS in comparison with PBMCs from a control group of healthy blood donors. The prevalence of HHV-6 variants was also studied in non-neoplastic and malignant lymphoproliferative disorders arisen in both HIV-infected and HIV-seronegative patients. The results were compared with those derived from a similar analysis, carried out in the same series of samples, concerning a reference herpesvirus, Epstein-Barr virus (EBV), which is involved in a subset of AIDS-related lymphoproliferative disorders [Levine, 1992; Boiocchi et al., 1993] and whose viral subtypes are distributed differentially in HIV-infected and HIV-seronegative individuals [Sculley et al., 1990; Boyle et al., 1991; De Re et al., 1993].

TABLE I. Frequency of HHV-6 Genome Detection in PBMCs From Healthy Individuals, From HIV-Seropositive Patients With Asymptomatic HIV Infection, LAS and Overt AIDS*

Source of PBMCs	$CD4^+$ cells ($\times 10^6$/L) median (range)	HHV-6-positive cases	HHV-6 variants A	HHV-6 variants B
Healthy blood donors	830 (480–1,315)	12/42 (29%)	3	9
Asymptomatic HIV-infected individuals	754 ± 205 (510–1,260) n.s.[a]	3/26 (11%)	—	3
HIV-seropositive patients with LAS	393 ± 229 (72–934) n.s.	1/13 (8%)	—	1
AIDS patients	69.1 (1–309) $P = 0.02$	1/20 (5%)	—	1

*Statistical evaluation was performed by comparing HHV-6 prevalence of PBMCs from each group of HIV-seropositive patients with that observed in healthy blood donors.
[a]n.s. = not significant.

MATERIALS AND METHODS

Study Materials

The presence of HHV-6 sequences was investigated in PBMCs from 26 asymptomatic HIV-seropositive individuals, 13 patients with LAS as the only clinical manifestation of HIV-infection, 20 AIDS patients and 42 healthy blood donors. Twenty LAS biopsies, 10 biopsy samples from 9 HD patients and 17 B-cell NHL specimens from HIV-infected individuals, all derived from an archival collection of frozen material, were also studied for the presence of HHV-6 and EBV genomes in comparison with 10 reactive lymphadenopathies, 43 HD and 35 NHL arisen in HIV-seronegative patients. HIV infection was demonstrated by multiple enzyme-linked immunosorbent assays (ELISA) for HIV-1 antibodies in serum and confirmed by Western immunoblot. The majority of HIV-infected patients were intravenous drug abusers. $CD4^+$ cell count was determined using standard methods. All pathologic samples were obtained from lymph node biopsies undertaken for diagnostic purposes and were stored frozen at −80°C until DNA extraction.

Histopathologic Analysis and Immunohistochemistry

LAS samples were classified according to the presence of hyperplastic or regressive changes of the lymphoid follicles, as reported previously [Öst et al., 1989]. The 10 non-neoplastic lymph node samples from HIV-seronegative patients were classified as chronic reactive lymphadenopathies. Specimens with malignant lymphoproliferative disorders were classified according to the Working Formulation for NHL [1982]. In all the cases, immunohistologic analysis of cryostat and paraffin-embedded tissue sections was performed using routine procedures, including the study of B and T cell-associated differentiation antigens, as described previously [Carbone et al., 1993]. The NHL samples from HIV-infected patients under study included 12 small noncleaved cell lymphomas (J group), 2 large-cell immunoblastic lymphomas (H group) and 3 $Ki\text{-}1^+$ anaplastic large-cell lymphomas [Boiocchi et al., 1993; Carbone et al., 1993] (*miscellaneous* group [The Working Formulation for NHL, 1982]). According to the Rye modification of the Lukes and Butler classification [Lukes et al., 1966], the 9 HIV-related HD cases investigated were: 5 of the mixed cellularity subtype, 2 of the nodular sclerosis subtype and 2 of the lymphocyte depletion subtype.

In all the LAS and other non-neoplastic lymph node samples, the immunohistochemical analyses for HIV p24 antigen and CD21 expression were carried out on Bouin-fixed, paraffin-embedded tissue sections. For HIV p24 antigen demonstration, the Kal-1 monoclonal antibody (MoAb; Dako A/S, Glostrup, Denmark) was used. Tissue sections were digested for 1 minute with Trypsin (0.33 mg/mL; Sigma, Milan, Italy), washed in ice-cold phosphate-buffered saline (2 × 5 minutes), placed in citrate buffer (pH 6) and irradiated in a microwave oven (Jet 900 W, Philiphs) twice for 5 minutes at 650 W. Immunostaining was performed by using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method [Cordell et al., 1984]. Immunohistochemical demonstration of CD21 expression was carried out by using 1F8 MoAb (Dako) and the avidin biotin peroxidase complex (ABC) method [Hsu et al., 1981] by using the ABC Elite kit supplied by Vector (Burlingame, CA).

Virologic Analyses

DNA purification and Southern blot analysis were carried out according to conventional methods. Ten micrograms of DNA from lymphoid biopsies were cleaved with *Hind*III restriction endonuclease and hybridized to radiolabelled HHV-6 DNA clone pHD10 [Martin et al., 1991]. The presence of HHV-6 and EBV sequences was investigated in DNA samples positive for PCR amplification for the β-globin gene fragment. PCR was always performed on 1 μg of DNA, corresponding to approximately 1.5×10^5 diploid cells. Particular care was taken to avoid contamination of PCR samples: DNA from normal and pathologic material was extracted in a laboratory not working with HHV-6 or EBV; blank reactions with no or unrelated DNA were interspersed among the samples, and different rooms were used to set up the reactions. The PCR assay was carried out at least twice for each specimen.

HHV-6 DNA sequences were sought by PCR using the set of primers described by Aubin et al. [1991], with 30

cycles of amplification (1 minute at 94°C, 45 sec at 60°C, 85 sec at 72°C) preceded by 5 minutes of denaturation at 94°C and followed by 5 minutes of final extension at 72°C. After electrophoresis on 2% agarose gel, the 832-bp amplified product was blotted on membrane and hybridized to a specific ^{32}P-labeled oligonucleotide [39] internal to the primers. After 12 hours of hybridization at 42°C in 6× SSC, 0.1% SDS, 0.2% Denhart solution, the membranes were washed at 42°C in 2× SSC, 0.1% SDS. The sensitivity of PCR amplification for HHV-6 was assessed using known amounts of the plasmid pZVH14, containing HHV-6 sequences amplified by the primers. Ethidium bromide staining of the aliquots of the reactions electrophoresed through agarose allowed the visualization of amplified products obtained from 1,000 target molecules, whereas following transfer on membrane and hybridization with a specific oligonucleotide, between 10 and 20 target sequences could consistently be detected. Characterization of the HHV-6 variants was performed by restriction endonuclease cleavage of the amplified product obtained after a nested PCR, maintaining the variant-specific restriction sites [Dewhurst et al., 1993]. After digestion of the amplified products with three different restriction enzymes (*Hind*III, *Hinf*I, *Taq*I), the results were compared to the pattern obtained from HHV-6 prototype strains: U1102 for variant A and Z29 and CV for the two typical profiles of variant B.

To amplify EBV sequences, PCR was carried out in a reaction mixture as described by Saiki et al. [1985]. After incubation at 95°C for 7 minutes, the reaction mixture was subjected to 35 cycles and to denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute. For a final extension, samples were kept at 72°C for 10 minutes. A 20-mer sense oligonucleotide spanning between bases 107,963 and 107,902 and a 28-mer antisense oligonucleotide spanning between bases 108,175 and 108,202 of the EBV internal repeat-3 (IR3) sequence [Baer et al., 1984] were used as primers. Following electrophoresis in 3.5% agarose gel, the amplified products were transferred to nylon membrane and hybridized at 55°C with a ^{32}P-labeled antisense oligonucleotide spanning between bases 108,062 and 108,102 of the EBV IR3 sequence [Baer et al., 1984]. After overnight hybridization, the membranes were washed at 59°C in 6× SSC and 0.05% Na pyrophosphate for 15 minutes. The sensitivity of this PCR protocol was assessed on serial dilutions of DNA from a recombinant plasmid containing EBV IR3 sequences. Following hybridization of Southern blots and overnight exposure of the autoradiograms, the assay allows to detect between 10 and 20 target molecules.

The characterization of the EBV subtypes was accomplished by a PCR amplification of the EBNA-2 region using primers specific for type 1 and type 2 EBV [De Re et al., 1993]. The amplification was preceded by an initial denaturation step at 95°C for 7 minutes and followed by a primer extension step at 72°C for 10 minutes. The PCR was carried out for 10 cycles of 1 minute at 94°C, 2 minutes at 55°C and 1 minute at 70°C followed by 40 additional cycles of 1 minute at 90°C, 1 minute at 55°C and 1 minute at 70°C. PCR products were analyzed subsequently by electrophoresis in 3.5% agarose gel stained with ethidium bromide and visualized by UV transillumination. The specificity of the amplified fragments was confirmed by Southern blot hybridization with type-specific ^{32}P-labeled oligonucleotide probes [De Re et al., 1993]. Analysis for PCR amplification of β-globin gene was carried out as described previously using RS79 and RS80 primers [Saiki et al., 1988].

The analysis of the physical conformation of EBV genome was undertaken by Southern blotting analysis, as described previously [Boiocchi et al., 1993]. The following probes were used: the 1.9-kb *Xho*I fragment corresponding to the *Eco*RI D fragment adjacent to EBV right terminal repeats and the 4.1-kb *Eco*RI fragment corresponding to the *Eco*RI region adjacent to EBV left terminal repeat [Raab-Traub and Flynn, 1986].

Statistical Evaluation

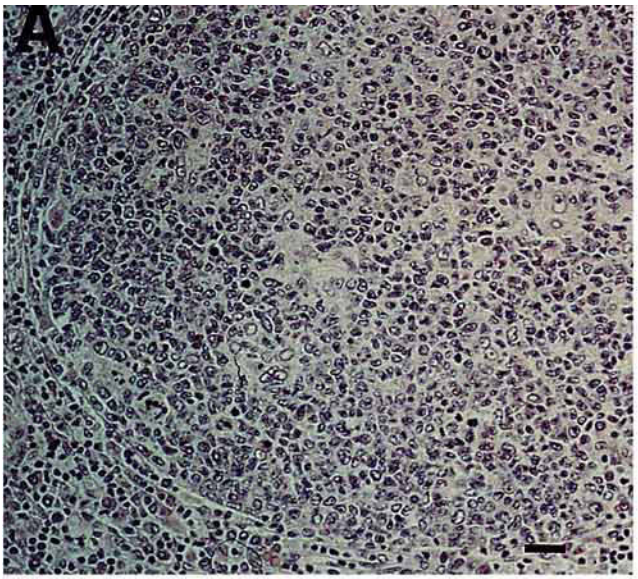
Statistical analyses were carried out using two-tailed Fisher's exact test.

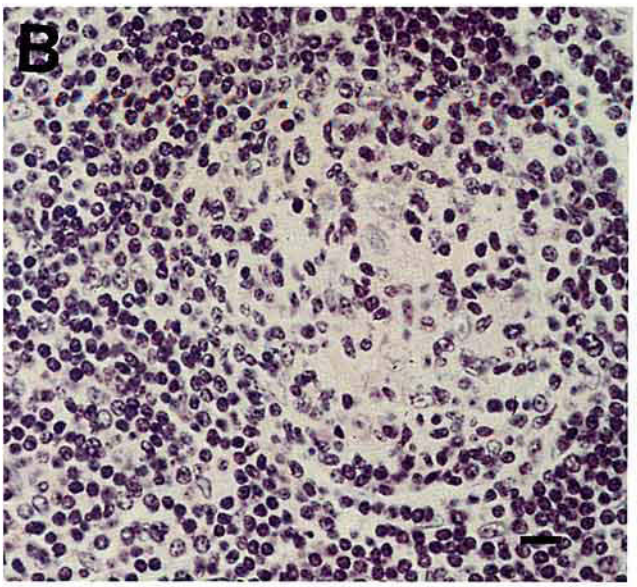
RESULTS

Due to the minimal amounts of viral sequences generally present in the samples investigated, the detection rate of HHV-6 depends greatly on the number of analyzed cells [Birx et al., 1986; Fairfax et al., 1994]. Therefore, for consistency's sake throughout the present study and to allow comparison among different samples and tissues, the same number of cells (1.5×10^5, corresponding to 1 μg of DNA) was always analyzed.

HHV-6 DNA was detected in 29% (12/42) of PBMCs from healthy donors (Table I). The prevalence of HHV-6 infection in PBMCs was lower in HIV-seropositive individuals with asymptomatic HIV-infection (3/26, 11%), the decrease was more evident in LAS patients (1/13, 8%) and was statistically significant in AIDS patients (1/20, 5%; $P = 0.02$; Table I). The efficiency of PCR was checked carefully throughout the study and, therefore, loss of sensitivity or presence of inhibitors could not account for the decreased presence of HHV-6 in PBMCs of HIV-infected individuals at different stages of the disease. The progressive reduction of HHV-6 DNA detection in PBMCs from the three groups of HIV-seropositive individuals correlated with the relative number of $CD4^+$ cells at the time of the analysis (Table I). As shown in Table I, in PBMCs from healthy blood donors HHV-6 variant B was three times more prevalent than variant A. In all HHV-6-positive PBMC DNAs from HIV-infected individuals, only variant B was detected (Table I) but due to the small number of patients in which HHV-6 sequences were detected no definitive conclusion on the distribution of HHV-6 variants in PBMCs from these patients could be drawn.

According to the classification of Öst et al. [1989], lymph nodes from HIV-infected patients with LAS revealed two main histopathologic patterns: follicular hyperplasia and follicular involution phases of the disease. No case showed aspects of follicular depletion. Follicular


A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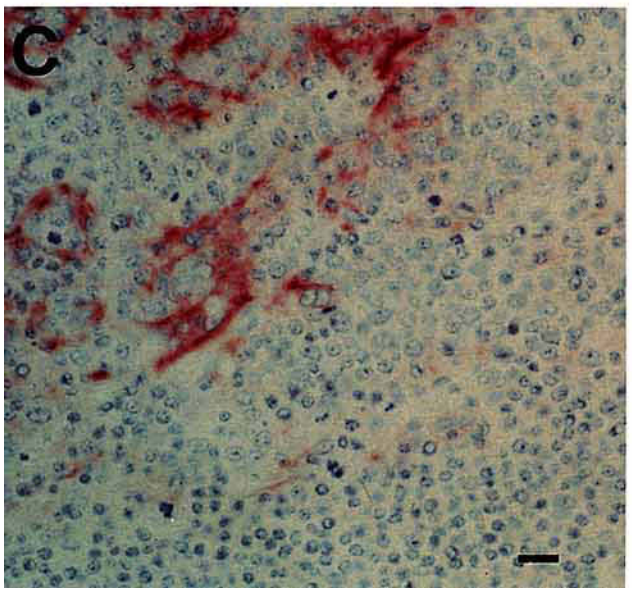
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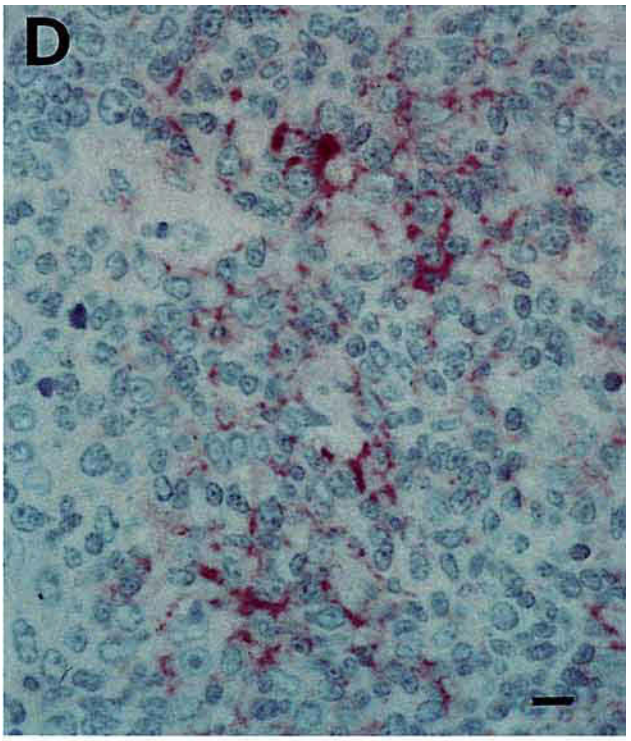
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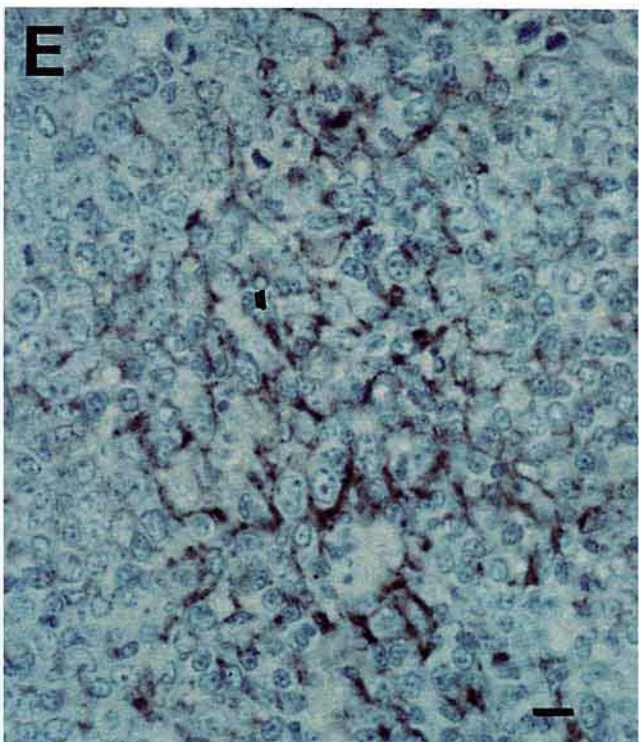
E

hyperplasia was found in 16 lymph nodes, whereas follicular involution, characterized by a hypervascular follicular pattern with small follicles, was found in the other 4 cases (Fig. 1). All lymph nodes from LAS patients displayed the staining of the follicular dendritic cell (FDC) network with anti-CD21 MoAb within the follicles. In all LAS specimens, except for one case, HIV p24 expression could be detected (Fig. 1). p24 positivity was restricted to the germinal centers and coincided with the staining of the FDC CD21-positive network (Fig. 1). In the p24-negative lymph node, in which a granulomatous reaction with necrosis was also present, some small follicles with the staining of the FDC CD21-positive network were observed. HHV-6 DNA sequences were detected in 13 of the 20 (65%) lymph nodes from LAS patients, whereas only 2 of the 10 (20%; $P = 0.02$) HIV-unrelated lymphadenopathies were HHV-6-positive (Table II and Fig. 2). In particular, HHV-6 was associated significantly with LAS showing follicular hyperplasia (13/16, 81%; $P = 0.003$). By contrast, the 4 cases with follicular involution were repeatedly HHV-6-negative (Table II) and the difference with respect to the LAS in hyperplastic phase was statistically significant ($P = 0.007$). Interestingly, at the time of the biopsy, the patients with HHV-6-positive LAS lymph nodes had remarkably higher $CD4^+$ cell counts (median value: 589, range 337–960 $\times 10^6$ $CD4^+$ cells/L) when compared with HHV-6-negative cases (median value: 325, range 72–950 $\times 10^6$ $CD4^+$ cells/L). Also the prevalence of EBV was higher in LAS (14/20, 70%) than in non-neoplastic lymph nodes from HIV-seronegative individuals (4/10, 40%), although the difference was not significant statistically (Table II).

All malignant lymphoproliferative disorders in HIV-infected patients were HHV-6-negative by Southern blot hybridization. HHV-6 DNA sequences were detected by PCR in 3 out of 10 HD (30%) and 1 out of 17 NHL (6%) specimens, frequency values similar to those observed in HD (12/43; 28%) and NHL (0/35) cases from HIV-seronegative patients (Table II and Fig. 2). Both the metachronous HD lesions from a single HIV-infected patient were HHV-6-negative. In contrast, EBV was detected at significantly higher frequencies in the same series of HIV-related HD (10/10; 100%) and NHL (9/17; 53%) biopsy specimens when compared with HD (15/43; 35%) and NHL (1/35; 3%) samples from HIV-seronegative patients (Table II). The study of the physical conformation of EBV genome carried out by Southern blot analysis revealed the presence of monoclonal EBV episomes in all HD (10/10) specimens and in 6 out of the 9 EBV-positive NHL from HIV-infected patients (data not shown).

Table III shows the results of the characterization of HHV-6 and EBV variants in this series of HIV-associated and HIV-unrelated lymphoproliferative disorders. All 13 HHV-6-positive LAS samples as well as the 2 HHV-6-positive HIV-unrelated lymphadenopathies carried variant B, whereas variant A was detected only in one LAS case as a coinfection with variant B (Table III and Fig. 3). Moreover, HHV-6 variant B was detected in the 3 HHV-6-positive HD from HIV-infected patients (in one of which together with variant A) and in 12 of the 13 HD cases from HIV-seronegative individuals (Table III). HHV-6 variant A was detected as a single infection in one HD case (Table III). The only HHV-6-positive NHL derived from a patient with HIV infection and carried variant B. While nearly all EBV-positive lymphoproliferative disorders from HIV-seronegative patients carried type 1 EBV, an almost equivalent distribution of the 2 EBV subtypes was observed in LAS, HD and NHL samples from HIV-infected patients (Table III).

DISCUSSION

Some recent studies showed that HHV-6 infection may spread to extralymphatic organs in terminally ill AIDS patients [Corbellino et al., 1993; Knox and Carrigan, 1994]. Nevertheless, the involvement of HHV-6 in the early phases of HIV infection, when HHV-6 could play a role in the progression of the disease, remains to be determined. The study of HHV-6 prevalence and distribution within different body districts in the early stages of HIV infection is necessary to assess the likelihood of in vivo interactions between HHV-6 and HIV. Aims of this study were to determine: (i) the prevalence and variant distribution of HHV-6 within the lymphoid system in HIV-infected and HIV-seronegative patients; and (ii) the site of possible interactions between HHV-6 and HIV in a clinical setting.

The first observation is that HHV-6 prevalence in PBMCs is lower in HIV-infected individuals than in healthy blood donors; the decrease correlates directly with the number of circulating $CD4^+$ T lymphocytes, confirming the findings of Fairfax et al. [1994]. Therefore, in contrast to other human herpesviruses [Birx et al., 1986; Laurence, 1990], the immune dysfunction taking place in the course of HIV infection does not seem to release HHV-6 from latency, at least during the early phases of the disease. It is not possible to draw conclusions with regard to the later stages of the disease, due to the low number of circulating $CD4^+$ cells, the main target of HHV-6 infection [Lusso et al., 1988]. Nevertheless, the low number of PBMC specimens positive for HHV-6 indicates that possibility of interaction between HHV-6 and HIV is relatively low in the peripheral blood of HIV-infected individuals.

A different picture stems from the examination of non-neoplastic lymph nodes, which showed that HHV-6 is more prevalent in LAS than in reactive lymphadenopa-

Fig. 1. Non-neoplastic lymph nodes from HIV-infected patients with LAS. **A:** Follicular hyperplasia: a large follicle with hyperplastic germinal center and extremely reduced mantle zone (hematoxylin and eosin, ×250). **B:** Follicular involution: a small follicular remnant without clearly defined mantle zone (hematoxylin and eosin, ×180). **C:** Paraffin-embedded section stained with anti-HIV p24 monoclonal antibody: p24 positivity is restricted to the follicular germinal center (APAAP method, ×250). **D** and **E:** Serial paraffin-embedded sections stained with anti-HIV p24 (D) and 1F8 (E) monoclonal antibodies: in a germinal center the irregular meshwork of p24 positivity (D) mimics that displayed by 1F8-positive follicular dendritic cells (E). (D: APAAP method, ×400; E: ABC method, ×400.)

TABLE II. Presence of HHV-6 and EBV DNA in HIV-Associated and HIV-Unrelated Lymphoproliferative Disorders*

Histology	HHV-6		EBV	
	HIV-seropositive	HIV-seronegative	HIV-seropositive	HIV-seronegative
Reactive lymphadenopathies	—	2/10 (20%)	—	4/10 (40%)
LAS (total)	13/20 (65%) $P = 0.02$	—	14/20 (70%) n.s.	—
LAS with follicular hyperplasia	13/16 (81%) $P = 0.003$	—	12/16 (73%) n.s.	—
LAS with follicular involution	0/4 n.s.[a]	—	2/4 (50%) n.s.	—
HD	3/10 (30%) n.s.	12/43 (28%)	10/10 (100%) $P = 0.0001$	15/43 (35%)
NHL	1/17 (6%) n.s.[a]	0/35	9/17 (53%) $P = 0.00005$	1/35 (3%)

*Statistical evaluation was carried out in each group of diseases by comparing the HHV-6 or EBV prevalence detected in HIV-associated and HIV-unrelated lymphoproliferative disorders.
[a]n.s. = not significant.

TABLE III. Distribution of HHV-6 and EBV Variants

Histology	HIV seropositivity	HHV-6-positive cases	HHV-6 variants			EBV-positive cases	EBV subtypes			
			A	B	A + B		1	2	1 + 2	Unt.[b]
Reactive LN[a]	HIV-positive (LAS)	13/20	—	12	1	14/20[c]	5	4	1	1
	HIV-negative	2/10	—	2	—	4/10	4	—	—	—
HD	HIV-positive	3/10	—	2	1	10/10	5	3	2	—
	HIV-negative	13/43	1	12	—	15/43	13	1	—	1
NHL	HIV-positive	1/17	—	1	—	9/17	5	2	1	1
	HIV-negative	0/35	—	—	—	1/35	1	—	—	—

[a]LN, lymph nodes.
[b]Unt., untypable.
[c]Characterization of EBV subtypes was performed on 11 of the 14 EBV-positive LAS samples.

thies from HIV-seronegative individuals. The association was statistically significant with LAS in the histologic phase of follicular hyperplasia ($P = 0.003$), while cases with follicular involution were HHV-6-negative. It is unlikely that HHV-6-carrying cells in LAS lymph nodes merely constitute circulatory elements present within the vascular tree. In fact, in analogy with other observations [Fairfax et al., 1994], we detected a marked decrease of HHV-6 prevalence in PBMCs of LAS patients. Moreover, HHV-6 DNA was detected with a very low prevalence in a large number of pathologic lymph nodes, which should not be different from LAS tissues with respect to vascularization. An attempt was made to quantitate the HHV-6 DNA molecules present in lymphadenopathies from both HIV-infected and HIV-seronegative patients by means of a competitive PCR assay developed recently (P. Mirandola et al., manuscript in preparation). However, all the positive samples carried fewer target molecules than the lowest detection limit of our competitive PCR, that is 20 target molecules in 150,000 diploid cells (data not shown). Therefore, it is unlikely that LAS samples carried cells actively infected with HHV-6, although this possibility cannot be entirely dismissed without searching for the presence of viral transcripts and proteins. Instead, the low amount of HHV-6 DNA in LAS could reflect increased levels of cells carrying latent HHV-6, either secondary to previous virus reactivation or due to the recruitment of lymphoid cells in the hyperplastic LAS lymph nodes. In accordance with this latter possibility, it should be noted that all LAS samples with follicular depletion and involution were HHV-6-negative. Therefore, the likelihood of HHV-6 reactivation could be higher in HIV-related than in HIV-negative lymphadenopathies due to either the increased number of latently infected cells and the immune suppression of HIV-infected patients.

During the early course of HIV infection, HIV accumulates in lymphoid organs [Embretson et al., 1993; Pantaleo et al., 1993], and all HHV-6-positive LAS from our series also carried HIV-infected cells. As shown by in vitro studies [Ensoli et al., 1989; Horvat et al., 1989; Lusso et al., 1989, 1991; Levy et al., 1990; Di Luca et al., 1991], the simultaneous presence of the two viruses could result in the increase of HIV replication and enhance the destruction of lymphoid cells. Our results suggest that the likelihood of interaction between HHV-6 and HIV is higher in lymph nodes than in peripheral blood. In fact, the prevalence of HHV-6 was markedly higher in hyperplastic lymph node biopsies (81%) than in PBMCs (8%) of LAS patients. Prospective studies are required however to determine whether patients with HHV-6-carrying LAS progress to AIDS more rap-

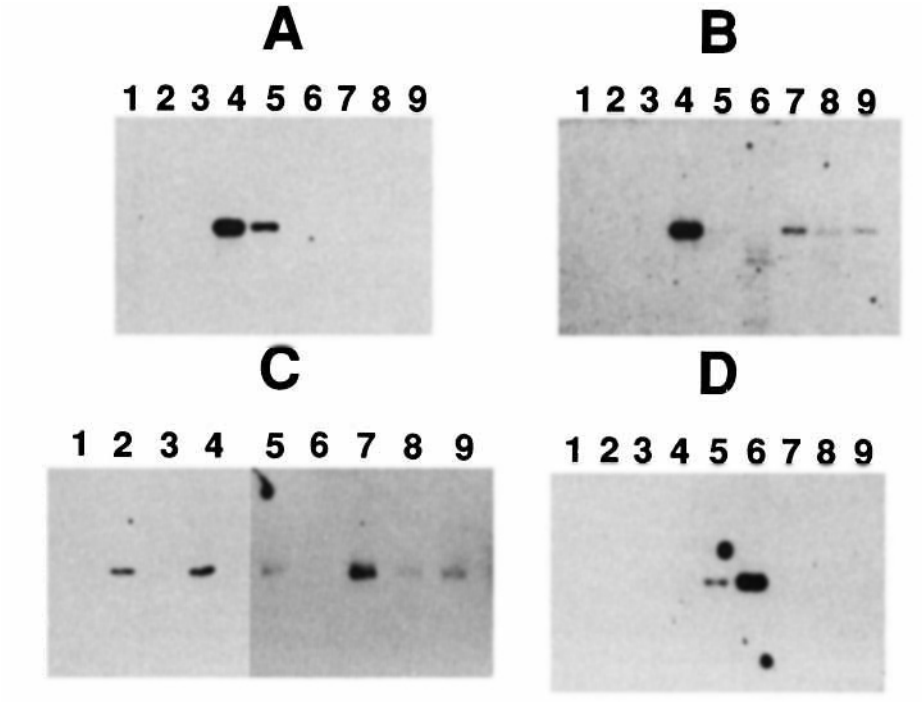

Fig. 2. Presence of HHV-6 in lymphoproliferative disorders from HIV-infected and HIV-seronegative patients. DNA extracted from biopsy specimens was amplified by PCR and subjected to agarose gel electrophoresis, Southern transfer, hybridization with a specific oligonucleotide and 72 hours of autoradiographic exposure. **A** shows the results of the analysis carried out in 9 HIV-unrelated reactive lymphadenopathies, 2 of which were HHV-6-positive. **B** presents the amplification of 9 HIV-associated lymphadenopathies, 4 of which carried HHV-6 DNA sequences. **C** shows the results of the analysis of 9 HIV-unrelated HD biopsies: 6 were HHV-6-positive and 3 HHV-6-negative. **D** presents the amplification of 9 HD biopsies from HIV-infected patients, 2 of which carried HHV-6 DNA sequences. All the positive samples yielded the expected hybridization band of 832 bp.

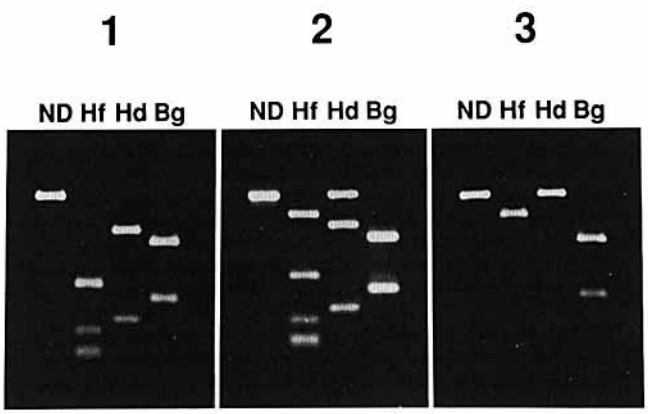

Fig. 3. Characterization of HHV-6 variants. The PCR-amplified product was analyzed by agarose gel electrophoresis without being digested (ND) or after cleavage with the restriction endonucleases *Hinf*I (Hf), *Hind*III (Hd) and *Bgl*II (Bg). **1** and **3** show the restriction pattern characteristic of HHV-6 variant A and variant B, respectively. **2** shows the pattern obtained from the study of an HIV-associated lymphadenopathy harboring both HHV-6 variants as indicated by the presence of bands typical of each variant.

idly than those with HHV-6-negative lymphadenopathies.

The possible association of HHV-6 with HIV-related malignant lymphoproliferative disorders was also investigated. HHV-6 DNA sequences were detected with a low prevalence in NHL arisen in HIV-infected patients (1/17, 6%) and only by PCR. This finding suggests that either HHV-6 was harbored by infiltrating normal lymphocytes or that viral infection took place after the expansion of the malignant clone, indicating that HHV-6 probably played no direct pathogenetic role. HHV-6 DNA was detected in 30% of HIV-associated HD cases and again only by PCR. Since in all of our HIV-related HD cases a monoclonal expansion of EBV-infected cells was documented by Southern blot analysis (data not shown), it is unlikely that HHV-6 is harbored by all EBV-carrying clonal cells, suggesting that either HHV-6 infects only a minority of Reed-Sternberg cells and their precursors, or is present in bystander infiltrating lymphoid cells. Further studies and in particular in situ hybridization analyses are needed to assess whether HHV-6 may contribute to the pathogenesis of HIV-related as well as of HIV-unrelated HD cases.

Interestingly, although most of LAS samples carried HHV-6 DNA sequences, a concomitant increase of HHV-6 prevalence in NHL and HD in HIV-infected patients was not detected. This contrasts markedly with the high prevalence of EBV observed in the same series of HIV-related lymphomas. These findings indicate that, unlike EBV [Shibata et al., 1991], the increased frequency of HHV-6 DNA in LAS samples does not constitute a significant risk factor for the development of HIV-associated malignant lymphoproliferative disorders.

Characterization of HHV-6 variant showed that HHV-6 B predominates in both HIV-infected and HIV-seronegative patients. Therefore, the context of HIV-related immune deficiency does not alter the distribution of HHV-6 variants, as observed with EBV, the prevalence of type 2 virus being markedly increased in HIV-infected patients.

Taken together, the results of the present study suggest that the number of HHV-6 latently infected cells increases in the early, hyperplastic phase of HIV-related lymphadenopathy, increasing the likelihood of HHV-6 reactivation. Since the presence of HHV-6 varies in the course of HIV disease progression, the possibility of interactions between the two viruses could also vary during the natural history of HIV disease and in the different body districts. HHV-6 has probably no direct role in the induction of HIV-associated malignant lymphoproliferative disorders. The HHV-6 variant distribution is not affected by HIV-associated immunosuppression, in contrast with what we observed for EBV.

HHV-6, therefore, could have different roles in the course of HIV infection: in the early phases, HHV-6 could behave as a cofactor or an inducing agent within the lymph nodes, while in the late stages of AIDS it could be responsible for the induction of severe systemic infections [Corbellino et al., 1993; Knox and Carrigan, 1994; Knox et al., 1995].

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